FORMULATION AND EVALUATION OF HERBAL MOUTHWASH CONTAINING EXTRACTS OF *IPOMOEA MARGINATA (DESR.) VERDC.* AS AN ANTIBACTERIAL AGENT TO CONTROL GINGIVITIS – AN IN-VITRO STUDY

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1. INTRODUCTION

Oral health is a significant determinant of general health, well-being, and overall quality of life. Gingivitis represents a widely prevalent chronic inflammatory condition affecting the oral cavity. Gingivitis is the most common cause for oral malodour and poor oral health. Dental plaque is a structurally and functionally organized biofilm that forms on the surface of teeth within few minutes after tooth brushing and is considered as the primary etiologic factor for gingivitis.[1] Dental plaque may be supragingival or subgingival plaque with considerable variation in their microbial composition. *Streptococcus mutans* is the predominant Gram-positive microorganism in supragingival plaque and the red complex pathogens namely *Porphyromonas gingivalis, Tannierella forsythia* and *Treponema denticola* appear later during biofilm development.[2] These bacteria trigger an inflammatory response and evoke a immune reaction leading to inflammation of the gingiva. Gingivitis frequently advances to periodontitis, which involves inflammation of the soft tissue and bone. Over time, persistent periodontitis has the potential to result in the loss of teeth.

There exists a hypothesis suggesting that low grade inflammation (LGI) may serve as a causal risk factor for chronic ailments, including diabetes mellitus, obesity, and cardiovascular diseases.[3] Therefore, it may be argued that biofilm bacteria serve as a covert contributing component in the emergence and advancement of chronic systemic disorders. The prevention of periodontal problems is a public health concern because it has been shown that these conditions contribute to the development of various systemic diseases.

Plaque control can be of two types, namely mechanical and chemical. Mouthwash or mouth rinse using chlorhexidine is a common form of chemical plaque

control. Chlorhexidine based antimicrobial agents have demonstrated moderate efficacy in eradicating dental plaque however, achieving consistent and targeted removal of these pathogens while preserving the natural microbial balance in the oral cavity remains a formidable task.[4] Bacterial resistance, change in oral microbiome and alteration in taste are some of the inherent side effects of chlorhexidine.[5]

Need for the study

One approach to address bacterial resistance is the utilization of herbal or natural products that include intricate chemical compositions thereby impeding the development of resistance in bacteria. The growing desire for novel antimicrobials has prompted numerous studies to focus on exploring the antimicrobial properties of phytochemicals derived from various plant species.

Plants have been used in treating ailments since time immemorial. 'Morning glory' is a commonly used plant in folk medicine that belongs to genus Ipomea, family Convolvulacea. The most common biologically active constituents from plants of genus Ipomoea are ergoline alkaloids, indolizidine alkaloids, nortropane alkaloids, phenolic compounds, coumarins, norisoprenoids, diterpene, isocoumarin and benzenoids, flavonoids and antocianosides, glycolipids, and triterpenes compounds. The remedial effects of plant materials are mainly due to the presence of substances called secondary metabolites of plants (SMoPs).[6,7]

Harnessing secondary plant metabolites would be a cost effective and innovative strategy to develop novel antimicrobials to combat the threat of antibiotic resistance.

With this background the present study aimed to explore the antimicrobial property and formulate a mouth wash from leaves of *Ipomea marginata.*

AIM AND OBJECTIVES

Aim

To formulate and evaluate an antibacterial herbal mouth wash containing extracts of *I.marginata*

Objectives

a) To extract and carry out phytochemical screening from leaves of *I.marginata*

b) To formulate a mouth wash from the leaf extract of *I.marginata*

c) To identify the minimal inhibitory concentration of bio-active compound on organisms causing gingivitis (*S.mutans* , *P.gingivalis* , *T.denticola and T. forsythia*)

2. REVIEW OF LITERATURE

Genus *Ipomea* is well known for its medicinal properties in different parts of the world. They are used to treat various conditions such as diabetes, hypertension, constipation, fatigue and arthritis. Some of the species showed antimicrobial, spasmolytic, hypotensive, anti-inflammatory activity and anticancer activity.

Sukitha *et al.*, 2022 in their study aimed to study enzymatic antioxidants (superoxide dismutase, catalase, polyphenol oxidase), nonenzymatic antioxidants (saponin, flavonoids, ascorbic acid and tannin), and anti-oxidant ability assays (total anti-oxidant, hydroxyl radical scavenging ability, reducing power activity, inhibition of lipid peroxide formation, DPPH scavenging activity and metal chelating assay) in *I. marginata* leaves and whole plant revealed that *I.marginata* possessed significant enzymatic anti-oxidants activities like superoxide dismutase, catalase, polyphenol oxidase in whole plant of *I. marginata*. plant. From the results of the study, it was confirmed that the whole plant of *I. marginata* showed strong antioxidant ability. Thus *I. marginata* whole plant can be used as a potential source of natural anti-oxidants.[8]

Haritha Potluri et al., 2021 in a study evaluated the antinociceptive activity of the methanolic extracts of *I.marginata* in rodents and observed that the methanolic extracts of *I.marginata* demonstrated significant antinociceptive activity and significant dose-dependent antinociceptive task at all doses. Isolated compound Ipalbidine demonstrated a significant docking score compared to standard diclofenac sodium. They concluded that *I.marginata* can be a potent resource of antinociceptive activity.[9]

Santosh Kumar *et al*., 2019 in their narrative review has mentioned the plant *I. marginata* has been used from time immemorial as an antidote to snake poisoning and treatment of skin infections.[10]

Sukitha *et al*.,2016 conducted a study to evaluate the phytochemical screening and antibacterial activity of the whole plant and the leaves of *I. marginata.* Antibacterial activity was evaluated against *S. aureus, Shigella sonnei, Enterobacter faecalis, Salmonella typhimurium, Micrococcus luteus, Vibrio cholerae, Klebsiella pneumoniae, Streptococcus pyogenes, E. coli, and Bacillus subtilis*. The observations of their study indicated methanol, acetone, benzene, water, and ethanol extracts of *I. marginata* exhibited significant antibacterial activity and further research can prove the antibacterial formulations can be produced from the plant.[11]

Research studies pertaining to *I.marginata* were limited hence relevant literature from plants of the same species are also cited.

Berenice Corona-Castañeda et L., 2013 in their study observed chloroform soluble extracts of flowers of Ipomea mucoides exerted a potentiation effect on clinically useful antibiotics against methicillin resistant Staphylococcus aureus (MRSA) by increasing their antibiotic susceptibility up to fourfold at concentration 25µg.[12]

Adsull *et al.,* 2012 in their study showed crude acetone extract of leaves of *Ipomea carnea* exhibited antibacterial activity against two strains, *Ptroteus vulgaris* and *Salmonella typhimurium* and ethanolic extract of leaves of *I.carnea* exhibited antibacterial activity against *Pseudomonous auroginosa*.[13]

3. MATERIALS AND METHODOLOGY

a. Collection of Plant material and authentication

With the guidance of local traditional healers, the plant was identified and the leaves were collected from Auroville forest (12.0052° N, 79.8069° E) Pondicherry, South India. The plant was authenticated by a Botanist, Professor Ayappan from French Institute of Pondicherry, Pondicherry, India. Voucher specimen was deposited in the Herbarium of French Institute of Pondicherry (Voucher specimen code: V.S. 001).

b. Extraction

The extraction was carried out using polar solvents of different polarity such as N hexane, ethanol and chloroform.

Ethanolic Extraction

The leaves of the plant were carefully cleaned, dried in the shade and ground into a powder using a warren blender. The extraction process employed a Soxhlet extraction equipment (Biocoction Manufacturing Pvt ltd, India). Within the apparatus's upper chamber, a porous thimble held 50 g of powdered leaf material. The lower boiling flask was filled with 200 ml of ethanol as a solvent. A heating mantle managed by a thermostat was used to raise the temperature of the flask to above 78^0 C. After heating the solvent to reflux, it was extracted. Following collection, the solvent extract was concentrated independently under low pressure. Following total evaporation, the residue's weight was recorded and saved. Complete extraction was ensured until a colourless liquid was collected at the top.

Chloroform Extraction

Fresh leaves of *I.marginata* were collected and dried in air at room temperature. The dried leaves were powdered. Powdered leaves were macerated with chloroform for 48 hours. Then the solution is filtered through Buchner funnel and collected. The chloroform is collected in a separate round bottom flask. The residual powdered leaves were then treated for extraction process with chloroform by Soxhlet extraction technique. The chloroform extract was then collected and mixed with the chloroform collected in the round bottom flask. The combined extracts were then concentrated to one tenth volume. The concentrated solution was then dried on a water batch. The residue is then subjected for the further evaluation.

N-Hexane Extraction

Fresh leaves of *I marginata* were collected and dried in air at room temperature. The dried leaves were powdered. Powdered leaves were macerated with n-hexane for 48 hours. Then the solution is filtered through Buchner funnel and collected the Hexane in a separate round bottom flask. The residual powdered leaves were then treated for extraction process with n hexane by Soxhlet extraction technique. The Hexane extract was then collected and mixed with solution collected in the round bottom flask. The combined extracts were then concentrated to one tenth volume. The concentrated solution was then dried on a water batch. The residue is then subjected for further evaluation.

c. Phytochemical screening

All the three extracts were subjected to preliminary phytochemical screening and tested for alkaloids, anthroquinones, flavonoids, phenols, steroils, tannins, terpenoids, cardiac glycosides, saponins, phlobatannin, reducing sugars, carbohydrates and protein/amino acids.[14]

d. Gas chromatography-Mass spectrometry (GC-MS)

GC-MS analysis of leaves of *I.marginata* was performed using a Perkin–Elmer GC Clarus 500 system comprising an AOC-20i auto-sampler and a Gas Chromatograph interfaced to a Mass Spectrometer (GC-MS) equipped with a Elite-5MS (5% diphenyl/95% dimethyl poly siloxane) fused with a capillary column (30 × 0.25 μm × 0.25 μm df). For GC-MS detection, an electron ionization system was operated in electron impact mode with ionization energy of 70 eV. Helium gas (99.999%) was used as a carrier gas at a constant flow rate of 1 mL /min, and an injection volume of 2 μL was employed (a split ratio of 10:1). The injector temperature was maintained at 250 °C, the ion-source temperature was 200 °C, the oven temperature was programmed from 110 °C (isothermal for 2 min), with an increase of 10 °C·min^{-1} to 200 °C, then 5 °C·min^{-1} to 280 °C, ending with a 9 min isothermal at 280 °C. Mass spectrum was taken at 70 eV; a scan interval of 0.5 sec and fragments from 45 to 450 Da. The solvent delay was 0 to 2 min, and the total GC-MS running time was 36 min. The relative percentage amount of each component was calculated by comparing its average peak area to the total areas. The mass-detector used in this analysis was Turbo-Mass Gold-Perkin-Elmer, and the software adopted to handle mass spectra and chromatograms was a Turbo-Mass version-5.2.[15]

Identification of Phytoconstituents

Interpretation on mass-spectrum GC-MS was conducted using the database of National Institute Standard and Technology (NIST) having more than 62,000 patterns. The spectrum of the unknown components was compared with the spectrum of known components stored in the NIST library. The name, molecular weight, and structure of the components of the test materials were ascertained.

Test Pathogens

The microbial strain used in the study were *S.mutans* (ATCC 700610), *P.gingivalis* (ATCC 33277), *T.denticola* (ATCC 35405) and *T.forsythia* (ATCC 43037) standard strains that were provided from HiMedia laboratories Pvt Ltd, Mumbai, India

Antibacterial Effect of Extract by Disc Diffusion Method

Twenty-four-hour old test pathogens were inoculated on Mueller-Hinton agar (HiMedia, laboratories Pvt Ltd, Mumbai, India) with blood at 0.5 McFarland optical density concentrations using sterile swab and allowed to dry for 15 min. Extract was prepared at 1mg/ml concentration and from this 25, 50, 75 and 100 µL was loaded on sterile discs. All the discs were placed over the agar surface inoculated with the test pathogen and incubated at 37± 2° C for 24-48 hour under anaerobic condition. Ethanol served as the negative control and the standard chlorohexidine was loaded on sterile disc to a concentration of 100 µG and served as the positive control. The zone of inhibition obtained after incubation was measured and recorded. The experiment was performed thrice in triplicates.

Minimum Inhibitory Concentration By Alamar Blue Oxidation Method

The minimum inhibitory concentration (MIC) of the extracts was evaluated using the broth dilution technique, with concentrations ranging from 100 to 0.78 µg/mL. MH broth was mixed with extract to make a final concentration of 200 µg/ml. Serial two-fold dilutions were prepared to make final concentrations ranging from 200 – 0.78 µg/mL. In a similar manner, chlorhexidine was also prepared to a concentration of. In each dilution, a volume of 10µL of bacterial solution containing 1×106 colony-forming units per millilitre (CFU/mL) was introduced. The dilutions were then subjected to incubation for a duration of 24 hours at a temperature of 37°C, while

maintaining anaerobic conditions. A viability test was used to evaluate the growth of the bacterial isolates in the test tubes after incubation. Fifty microliters of resazurin were added to the tubes, which were then incubated for a duration of 15 minutes. The process of dye reduction was documented. The maximum dilution at which the dye does not undergo reduction was determined as MIC.

Time-Kill Kinetics Assay

100 mL of mouth wash made at the MIC level was combined with 1 mL of an inoculum size of less than 300 CFU/mL, and it was then incubated at 37°C. At 0, 6, 12, 18, and 24 hours, 1.0 mL aliquots of the medium were obtained, aseptically plated onto 20 mL of nutritional agar, and then incubated for 24 hours at 37°C. The colony forming unit (CFU) was recorded. Three separate experiments were conducted in triplicate to complete the protocol, and the log CFU/mL was plotted against time on a graph.[16]

TLC direct bioautography

To enable identification of biologically active constituents in the plant mixture direct bioautography was carried out. The inoculum was prepared by growing an overnight culture of the test bacteria and the next day the turbidity was matched to McFarland 0.5 standard. The inoculum was spread onto MH agar plate and the plates were swabbed with the inoculum of representative bacterial strains in the direct bioautography procedure. Sterile lens paper was used to cover the seeded MH agar plate, aseptically and the dried TLC plates with corresponding spots were layered on top of it. The TLC plate was placed face down with the silica-coated side evenly in contact with the lens paper, and it was incubated for 24 h at 35 ± 2°C. The TLC plate readings were compared and the zone of inhibition was observed. The fractions were gathered, placed on sterile discs, and utilized to confirm the active fraction.[17]

Gas chromatography-Mass spectrometry (GC-MS) of Active fraction

The active fraction obtained from direct TLC directed bioautography was subjected to GC-MS to identify the volatile constituents. Phenol, 3,5-bis(1,1-dimethylethyl)- and triethyl citrate were two compounds eluted in the active fraction.

Fourier Tansform Infrared Spectroscopy (FTIR)

Fourier transform infrared (FTIR) spectroscopy is an instrument able to detect the functional groups of extract. Pellets for infrared analysis were obtained by grinding 1 mg of dried extract with 100 mg of dry potassium bromide, and the mixture was pressed into a 16 mm diameter mould. The FTIR spectra were recorded on a Paragon 1000, Perkin-Elmer spectrometer (4,000-400 cm-1, 2 cm-1 of spectral resolution, and 32 scans).

Molecular docking analysis

The bioactive compounds isolated by TLC directed bioassay was proceeded for the molecular docking studies to determine the mode of action and binding efficacy involved in the molecular level to predict possible active sites.

Target protein identification and preparation

The three-dimensional structure of the target virulence proteins (Mirolysin, Glucansucrase, dipeptidyl peptidase III, Cystalysin) were retrieved from the Research Collaborator for Structural Bioinformatics (RCSB) Protein data bank (www.rcsb.org) respectively. A chain of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase (HMGR) was pre-processed separately by deleting other chains (B, C and D), ligand, as well as the crystallographically observed water molecules (water without hydrogen bonds). Using Pymol software, water molecules and ligands already

present in the proteins were removed; hydrogen atoms were added and saved in Protein Data Bank (PDB) file format.

Prediction of active site

Prediction of the active site is important in structure-based drug design. Co-ordinates of binding sites of the proteins were identified using the software UCSF chimera Docking Molecular docking calculations were carried out with the aid of the software AutoDock version 4.2 and binding energy of the protein—ligand adducts were obtained.

Formulation of mouthwash from ethanolic extracts of *I.marginata*

50g of dried plant powder extracted with ethanol using Soxhlet and concentrated under vacuum dryer. About 100 mg of dried sample dissolved in 100 ml water and filtered. 20g of sodium saccharin was added in 100 ml water as sweetener. 50 mL of glycerol was mixed in 100 water to keep the moisture along with 50 g sorbitol. All the solutions were mixed and the final total volume is made up to 10000 mL with distilled water. All the ingredients were added under magnetic stirrer (100rpm) at room temperature in a biosafety cabinet and stored in amber bottle.

Accelerated stability studies

Based on the ICH guidelines accelerated stability studies was carried out with parameters such as colour, odour, foam, taste, phase separation, Ph, turbidity, viscosity, sedimentation, precipitation, homogeneity, specific gravity, density, phenolic content and surface tension.

Cytotoxicity of cells

To 1 ml of normal saline 200ul of RBC suspension was added and used as reaction base. Different volume of mouth wash (25, 50, 75 and 100 µL) were added to

RBC suspension and incubated for 4h. 1 % SDS used as positive control. Normal saline used as negative control. After 4 h tubes were centrifuged and the OD of aqueous phase was taken at 560 nm. The percentage of haemolysis was calculated then by the formula OD of test- negative control / negative control x 100.[18]

Heavy metal detection

Heavy metal detection in the sample was done by Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES) method of analysis.[19]

IV. Results and Discussion

Genus ipomea has long been used for medicinal purposes chiefly because of the secondary metabolites present in the leaves.

After hot soxhlet extraction of the leaves of *I marginata* the preliminary phytochemical study revealed that ethanolic extract contained alkaloids, flavonoids, phenols, sterols, tannins, terpenoids, cardiac glycosides, phlobatannin, reducing sugars, carbohydrates and protein/amino acids. [Table 1].

Table 1: Phytochemical constituents of ethanolic extract of leaves of *Ipomea marginata*

S.No	Phytochemical	Chloroform	Ethanol	N Hexane
1	Carbohydrate	Positive	Positive	Positive
2	Glycoside cardiac	Negative	Positive	Positive
3	Glycoside	Negative	Positive	Positive
4	Alkaloid	Positive	Positive	Positive
5	Flavonoid	Positive	Positive	Negative
6	Terpenoid	Positive	Positive	Negative
7	Phenols and Tannins	Negative	Positive	Negative
8	Saponins	Negative	Negative	Negative
9	Sterols	Negative	Positive	Positive
10	Phlobotanins	Positive	Negative	Positive
11	Anthraquinones	Positive	Negative	Positive

Presence of phytochemicals in leaves are the most important reason for manifestation of their biological activities. After the extraction with three different solvents based on their polarity it was seen on initial phytochemical screening ethanolic extract exhibited presence of alkaloids, flavonoids, phenols and tannins which have proven to be the cause for significant antibacterial property.

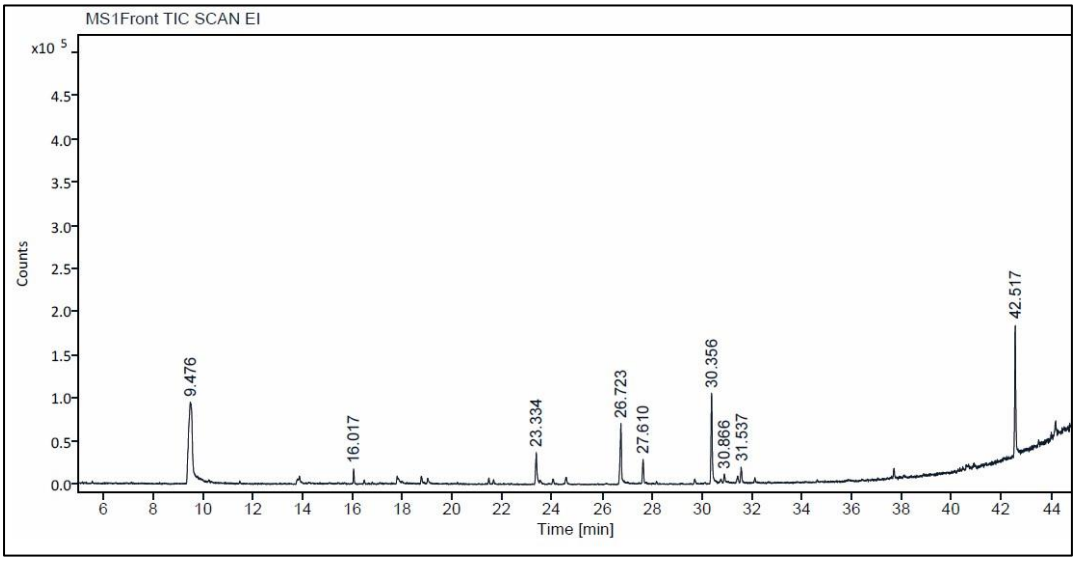
GC-MS Analysis

The steam distillation of ethanolic extract of leaves of *I marginata* was subjected to GC-MS analysis and the results are summarized in Table 2

Table 2: Volatile components identified in the ethanolic extract of *I marginata* by gas chromatography coupled to mass spectroscopy

S.NO	Compound Name	Abundance (%)	Retention Index
1.	2,3-Butanediol, 1,4-dimethoxy-	38.54	1094
2.	Ethyl 4-oxo-2-phenylpentanoate	42.72	1629
3.	Neophytadiene	22.31	1840.6
4.	n-Hexadecanoic acid	75.94	1968.2
5.	Hexadecanoic acid, ethylester	66.43	1993.5
6.	Phytol	49.35	2113.9
7.	3-Tridecen-1-yne, (Z)-	12.2	1319
8.	9,12,15-Octadecatrienoic acid, (Z,Z,Z)-	9.7	2154
9.	Squalene	26.28	2835.8

Figure 1: Chromatogram of volatile compounds eluted from ethanolic extract of leaves of *I.marginata*



Antibacterial activity

Zone of Inhibition

With regard to *S.mutans* the mean zone of inhibition (ZOI) was found to be more effective for herbal extract (18±0) compared to chlorhexidine mouth wash (12±1) (Figure 2a). This difference was also found to be statistically significant (p= 0.00024). With respect to *P. gingivalis* also the mean zone of inhibition (ZOI) was found to be more effective for herbal extract (18.3±0.57) compared to chlorhexidine mouth wash (28.3±0.577) (Figure 2b) and (p=0.00001) with respect to *T.denticola* the mean zone of inhibition (ZOI) was found to be more effective for herbal extract (10.3±0.57) compared to chlorhexidine mouth wash (0) (Figure 2c)with (p=0.00001) and with respect to *T.forsythia* the mean zone of inhibition (ZOI) was found to be statistically significant for herbal extract (14±1.73) compared to chlorhexidine mouth wash(18±1) (Figure 2d) with (p=0.0128) (Table 3).

Table 3: Zone of Inhibition (in mm) of Herbal Extract and Chlorhexidine at Different Concentrations

Test Pathogens	10 µG	25 µG	50 µG	Herbal Extract 100 µG	Chlorohexidine (100 µG)	t statistic	p value
S.mutans	12.33±0.57	13.3±0.57	16±1	18±0	12±1	10.392	0.00024
P.gingivalis	0	0	0	18.3±0.57	28.3±0.577	21.213	0.00001
T.denticola	5±1	5±1	5±1	10.3±0.57	0±0	31	0.00001
T.forsythia	5±1	5±1	5±1	14±1.73	18±1	3.464	0.0128

Figure 2: Zone of Inhibition of herbal extract versus 0.2% Chlorhexidine

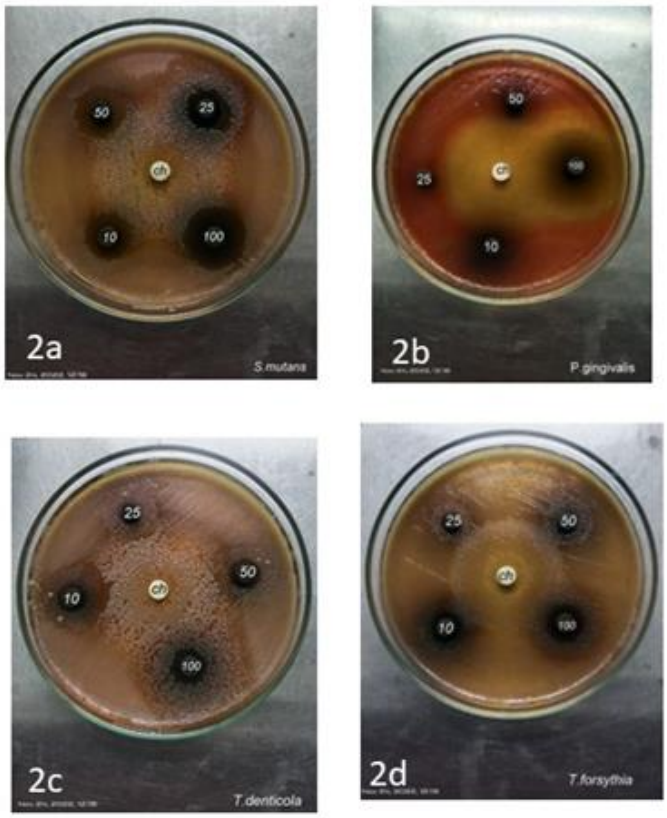


Figure 2a: Zone of inhibition of herbal extract and 0.2% CHX mouth wash for S mutans

Figure 2b: Zone of inhibition of herbal extract and 0.2% CHX mouth wash for *P gingivalis*

Figure 2c: Zone of inhibition of herbal extract and 0.2% CHX mouth wash for *T denticola*

Figure 2d: Zone of inhibition of herbal extract and 0.2% CHX mouth wash for *T forsythia*

Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentration (MIC) for the herbal extract for *S.mutans, P.gingivalis, T.denticola and T.forsythia* was 6.25µg/mL, 100µg/mL, 100µg/mL and 50µg/mL respectively and that standard chlorhexidine was 25µg/mL, 12.5µg/mL, 200µg/mL and 100µg/mL respectively (Table 4). The MIC values determine the levels of susceptibility or resistance of a specific bacterial strain to a particular drug and drugs with lower MIC scores were more effective antibacterial agents. In the present study the herbal extract was found to be more effective against *S.mutans, T.denticola* and *T.forsythia* compared to chlorhexidine mouth wash. However, it was found chlorhexidine mouth wash was more effective against *P.gingivalis* compared to the herbal extract (Table 4).

Table 4: MIC of Herbal Extract and Chlorhexidine

Test Pathogens	MIC of Herbal Extract(µg/ml)	MIC of CHX(µg/ml)
S.mutans	6.25	25
P.gingivalis	100	12.5
T.denticola	100	200
T.forsythia	50	100

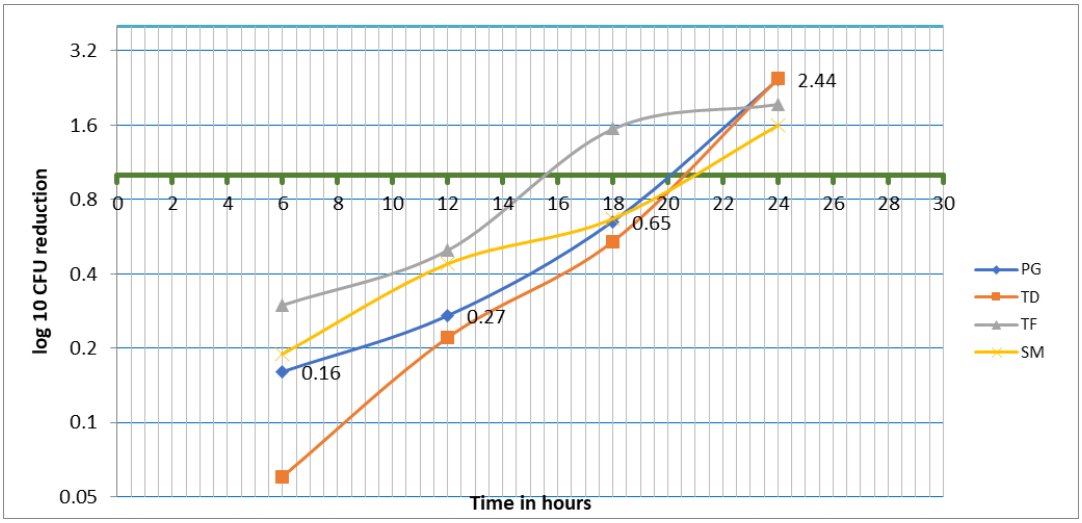
Time kill kinetics

The results obtained for the time-kill study of test pathogens tested at MIC concentration is displayed in the growth curve in the form of log of colony forming unit (Figure 3). The reduction in colony forming units (CFU) over a period of 24 hours showed 97.5% reduction of *S.mutans*,99.65% reduction of *P.gingivalis*, 99.67% reduction of *T.denticola* and 99.8% reduction of *T.forsythia* (Table 5).

Table 5: Reduction in CFU of test pathogens (%) at different time intervals

Test Pathogens	Percentage reduction (%)			
	6 hours	12hours	18hours	24hours
S.mutans	36	64	79	97.5
P.gingivalis	31	47	77.8	99.65
T.denticola	13	40	71	99.67
T.forsythia	51	68.8	97.2	99.8

Figure 3: Time Kill Curve of Test pathogens (PG:*P gingivalis*;TD:*T denticola*;TF: *T forsythia*; SM: *S mutans*)



Time kill kinetics assay in the present study showed the herbal extract demonstrated bactericidal effect in a time dependent manner and the viability of pathogens was abolished within 24 hours in the time-killing test for the 5 log cycle reductions were detected. The findings of our study align with previous research which also observed a significant bactericidal impact when the concentration of the antimicrobial agent was doubled (2 × MIC) and the duration of contact was extended. Different bacteria and plant extracts exhibited varying degrees of time-dependent microbial inhibition, according to the results of the time killing experiment. Response

to microbial infection by plant secondary metabolites can be viewed as one of the contributing factors for antimicrobial properties exhibited by plants.

TLC Direct bioautography

Bioassay results revealed *S. mutans* and *T. denticola* were resistant to Fractions 1 and 2; however, Fraction 3 demonstrated sensitivity against both the organisms. It was observed the zone of inhibition for *S.mutans* and *T. denticola* was 16 mm. 0.2% CHX was used as positive control and the zones of inhibition for positive control for *S. mutans* was 16 mm (p=0.013) and for *T. denticola* was 17 mm (p=0.001) respectively.(Table 6) (Figure 5)

Direct contact bioautography demonstrated 3 fractions; namely, Fraction 1, Fraction 2 and Fraction 3 corresponding to Rf values 0.71, 0.79, 0.85 at 254 nm (Table 7) (Figure 4)

Table 6: Zone of inhibition (mm) of three fractions and chlorhexidine by direct bioautography

Pathogens	Fraction 1	Fraction 2	Fraction 3 (mm)			Ethanolic extract (100µg/ml) (Mean±SD)	Chlorhexidine (Mean ± SD) (100µg/ml)	*p* value
S.mutans	0	0	16	15	15	15.6	16	0.013
T.denticola	0	0	16	16	17	16.3	17	0.001

Table 7: Rf values of the fraction at 365 nm and 254 nm

Fraction	Absorption at 365 nm	Absorption at 254 nm
1	0.71	0.71
2	-	0.79
3	-	0.85

Figure 4: Chromatography at 365 nm and 254 nm

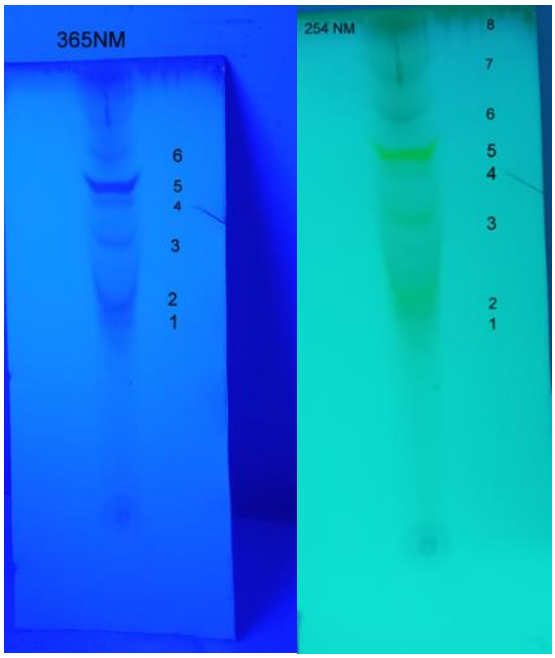
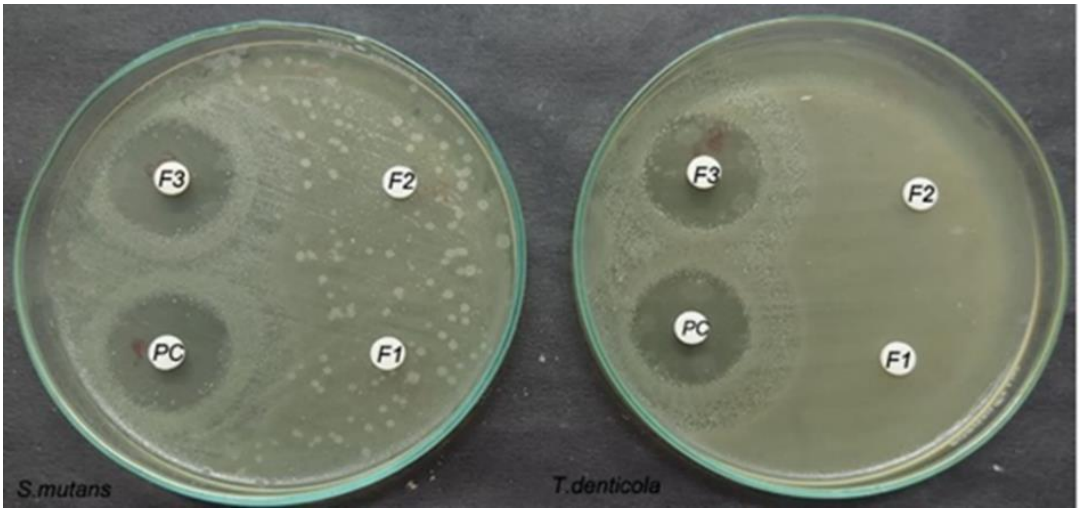


Figure 5: Zone of Inhibition of fraction 1(f1),fraction 2(f2),fraction 3(f3) and positive control chlorhexidine (PC)



GC-MS of active fraction

The active fraction obtained after TLC direct autobiography was subjected to GC-MS. Two peaks were observed in the chromatogram first peak corresponded to phenol, 3,5-bis (1,1- dimethylethyl) and second peak triethyl citrate. (Table 8) (Figure 6)

Figure 6: Chromatogram of Eluted Compounds from Active Fraction

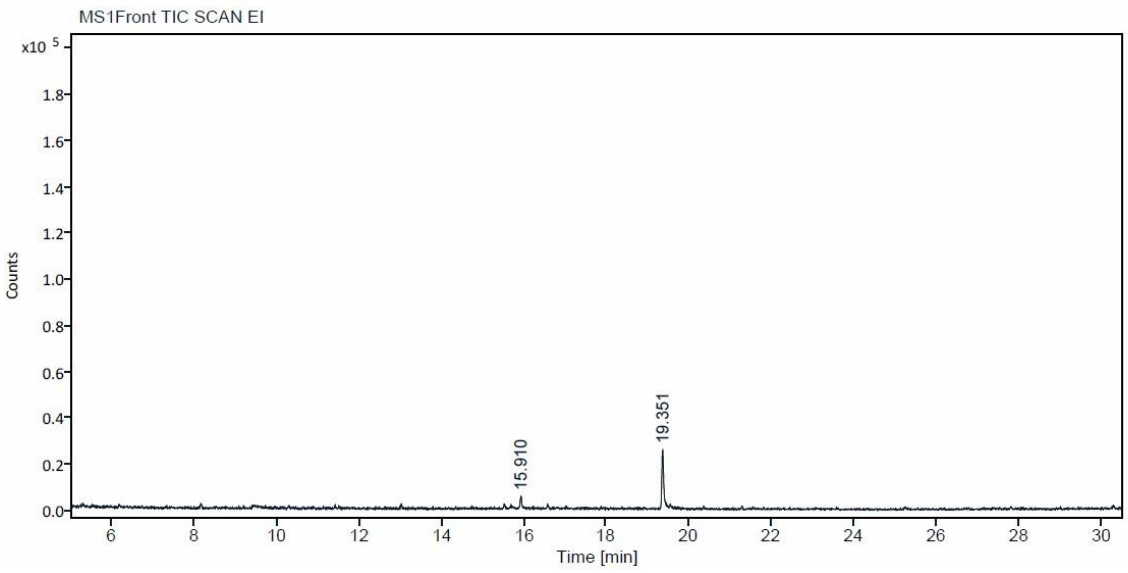


Table 8: Volatile compounds eluted by GC-MS of the active fraction

S.no	RT	Compound name	Molecular wieght	Molecular formula
1	15.910	Phenol, 3,5-bis (1,1-dimethylethyl	206.3239	$C_{14}H_{22}O$
2	19.351	Triethyl citrate	276.2830	$C_{12}H_{20}O_7$

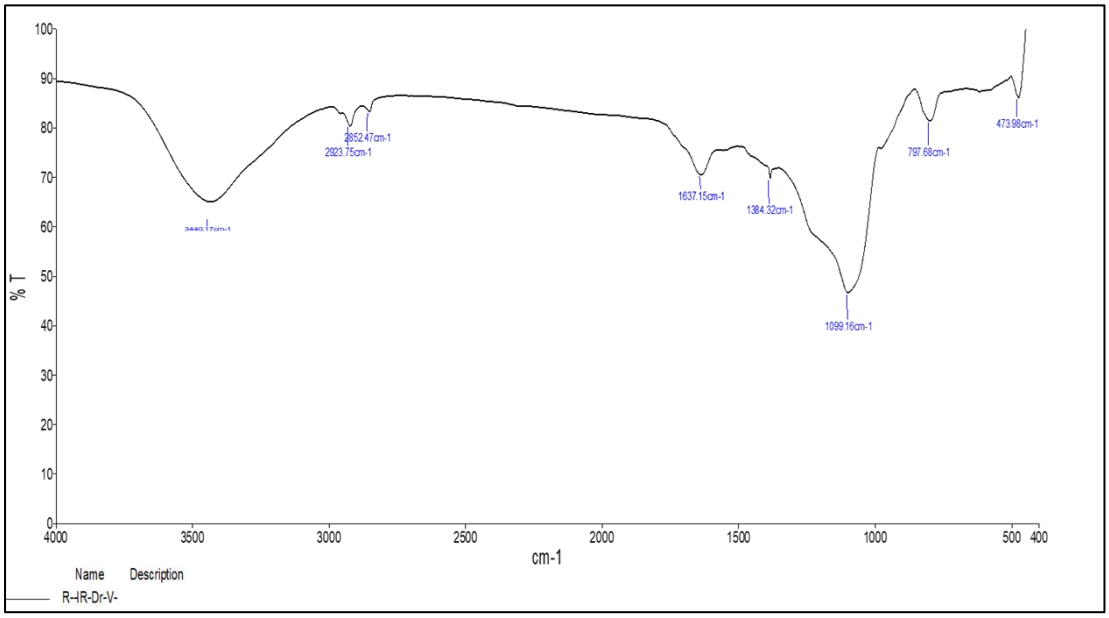
Fourier Tranform Infrared Spectroscopy (FTIR)

FTIR was done to identify the functional group. Two bands appear at 2923 and 2852 cm-1, which is due to carboxylic acid stretching frequency. A sharp band appeared at 1630 cm-1 can be attributed to the ν (-C=C) stretching due to alkene group of the compound. Vibration at 1384 cm^{-1} denotes C-H bending corresponding to aldehyde. C=C bending of alkene group was recorded at 799 cm^{-1}. The above observations denote the presence of functional OH group in the extract suggesting a phenolic compound as evidenced by the GC-MS report wherein phenol, 3,5-bis (1,1-dimethylethyl) was eluted. The FTIR spectrum was matched to NIST library and confirmed the IR spectrum of phenol, 3,5-bis (1,1- dimethylethyl)(Table 9)(Figure 7)

Table 9: Vibration of different functional groups

Group	O-H stretching	O-H stretching Carboxylic acid	C-C stretching Alkene	C-H bending aldehyde	C-O stretching Secondary alcohol	C=C bending alkene
Vibration cm^{-2}	3440	2923	1630	1384	1099	799

Figure 7: FTIR of different functional groups



In silico molecular docking analysis

Mirolysin is a secretory protease of *T.forsythia* docked with isolated compound and compared with standard. The interaction of Phenol, 3,5-bis(1,1-dimethylethyl)- showed docking score -5.7. ASN amino acid showed hydrophobic interaction with ligand C6 and C13 atom. The Oxygen atom of O1 interacted with proline residue and formed hydrogen bond. Triethyl citrate showed hydrophobic and hydrogen bond formation between O1- TYR with affinity of -5.3cal/mol. Likewise C14 and C15 atom of Chlorhexidine interacted with ASN and the docking score was -7.4 kcal indicates that it has superior specificity. In addition to that standard showed formation of ionic interaction, pi-pi stacking and hydrophobic interaction with ASN of Mirolysin.

Glucansucrase secreted by *S.mutans* creating adherent environment for structural bacteria colony forming dental biofilm. The target is docked with isolated compound and compared with standard. The interaction of Phenol, 3,5-bis(1,1-dimethylethyl)- showed docking score -6.2 kcal/mol with one hydrogen bond

hydrophobic interactions. The carbon atom of Phenol 3,5-bis(1,1-dimethylethyl)- interacted with ASN and SER and formed hydrophobic interaction. Docking of Triethyl citrate showed binding affinity -6.2 kcal/mol by forming hydrophobic interaction and no hydrogen bonds were detected. The docking score of Chlorhexidine was -8.3 kcal/mol. It formed hydrogen bond with ASN, PRO and GLN. Further it showed ionic interaction, hydrophobic contact with PRO (N5,N7) and ASP (C4,10, 21).

Dipeptidyl peptidase III which is secreted by *P.gingivalis* docked with isolated compound and compared with standard. The interaction of Phenol, 3, 5-bis(1,1-dimethylethyl)- showed docking score -6.0 kcal/mol along with hydrophobic and pi-pi stacking against ASN. The oxygen atom of Phenol 3, 5-bis (1,1-dimethylethyl)- interacted with ARG and TYR and formed hydrogen bonding. Interaction of Triethyl citrate with dipeptidyl peptidase III shows O1, O7 and O6 atoms interacted with TYR, ASP and GLN and hydrogen bond having affinity of-5.4 kcal/mol. The docking score of Chlorohexidine was -8.3 kcal/mol. It formed hydrogen bond with ALA (N8) and PHE (N8, N4).

Cystalysin1C7O secreted by *T denticola* docked with isolated compound and compared with standard. The interaction of Phenol, 3,5-bis(1,1-dimethylethyl)- showed docking score -5.9 kcal/mol indicates moderate binding efficiency. The atom of Phenol 3,5-bis(1,1-dimethylethyl)- interacted with VAL and LEU formed 2 hydrogen bond, pi-pi stacking with LEU and hydrophobic interaction with LYS. The docking score of Triethyl citrate was -4.8 with hydrogen bond towards SER and LYS whereas Chlorhexidine was -7.4 kcal/mo indicates that it may have superior

specificity than the isolated compound. It formed Hydrogen bond with TYR and hydrophobic interaction with VAL.

A similar in silico study was conducted to assess the antinociceptive activity of the methanolic extract of *I.marginata* by autodock 4.0 and date warrior software applications, results of the study revealed Ipalbidine compound isolated from methanolic extract of *I.marginata* demonstrated a very good docking score of -8.26 compared to standard diclofenac which showed a docking score of -7.03 guaranteeing a good binding compatibility among the ligand and receptor.[9]

Table 10: Docking score of ligand Phenol, 3,5-bis(1,1-dimethylethyl),triethyl citrate and chlorhexidine with protein Microlysin

Protein	Ligand	Score Kcal/mol	Ligand-Amino acid
Mirolysin 6R7W	Phenol, 3,5-bis(1,1-dimethylethyl)-	-5.7	O1-PRO 6
	Triethyl citrate	-5.3	O1 TYR
	Chlorhexidine	-7.4	C14-ASN C15-ASN

Table 11: Docking score of ligand Phenol, 3,5-bis(1,1-dimethylethyl), triethyl citrate and chlorhexidine with protein Glucansucrase

Protein	Ligand	Score Kcal/mol	Ligand-Amino acid
Glucansucrase 3AIC	Phenol, 3,5-bis(1,1-dimethylethyl)-	-6.2	O1-PRO
	Triethyl citrate	-6.2	Hydrophobic PRO
	Chlorhexidine	-8.3	N9-ASN N1-PRO N8-GLN

Table 12: Docking score of ligand Phenol, 3,5-bis(1,1-dimethylethyl)-,triethyl ethyl citrate and chlorhexidine with protein dipeptidyl peptidase III

PDB ID	COMPOUND	Score Kcal/mol	LIGAND- AMINO ACID
dipeptidyl peptidase III 6JTB	Phenol, 3,5-bis(1,1-dimethylethyl)-	-6	O1- ARG/TYR
	Triethyl citrate	-5.4	O1-TYR AO7- ASPO6-GLN
	CHLOROHEXIDINE	-8.3	N8-ALAN4/7 -PHE

Table 13: Docking score of ligand Phenol, 3,5-bis(1,1-dimethylethyl)- , triethyl ethyl citrate And chlorhexidine with cystalysin.

Protein	COMPOUND	Score Kcal/mol	LIGAND- AMINO ACID
Cystalysin (1C7O.B)	Phenol, 3,5-bis(1,1-dimethylethyl)-	-5.9	C8-LEU/ N4- VAL
	Triethyl citrate	-4.8	O5-SER O2-LYS
	CHLOROHEXIDINE	-7.4	C17- TYR

Figure 8: Three dimensional interaction of Microlysin with Phenol, 3,5-bis(1,1-dimethylethyl

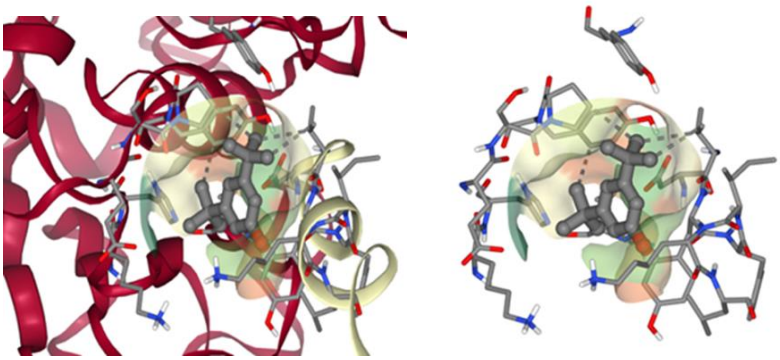


Figure 9: Three dimensional interaction of Glucansucrase with Phenol, 3,5-bis(1,1-dimethylethyl

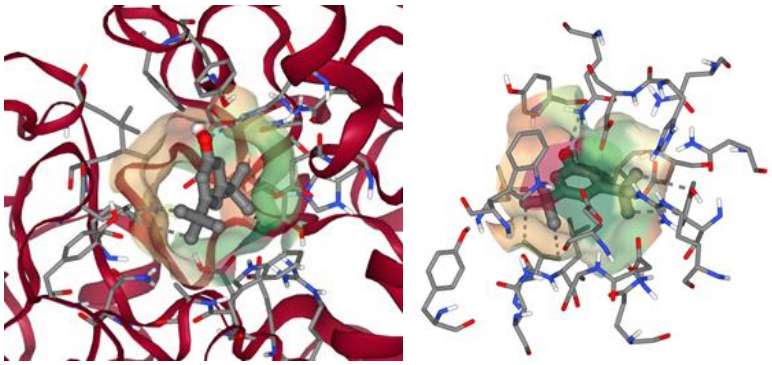


Figure 10: Interaction of Dipeptidyl peptidase III with Phenol, 3,5-bis(1,1-dimethylethyl)

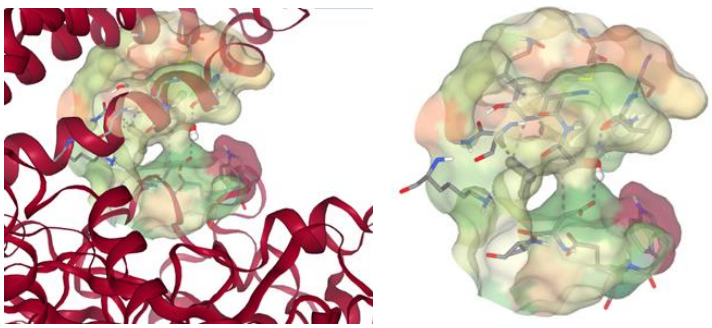
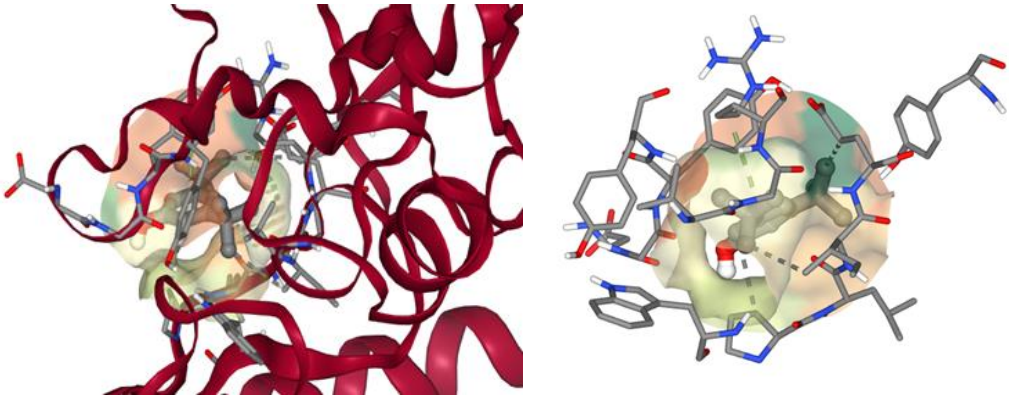


Figure 11: Interaction of Cystalysin1C7O with Phenol, 3,5-bis(1,1-dimethylethyl)



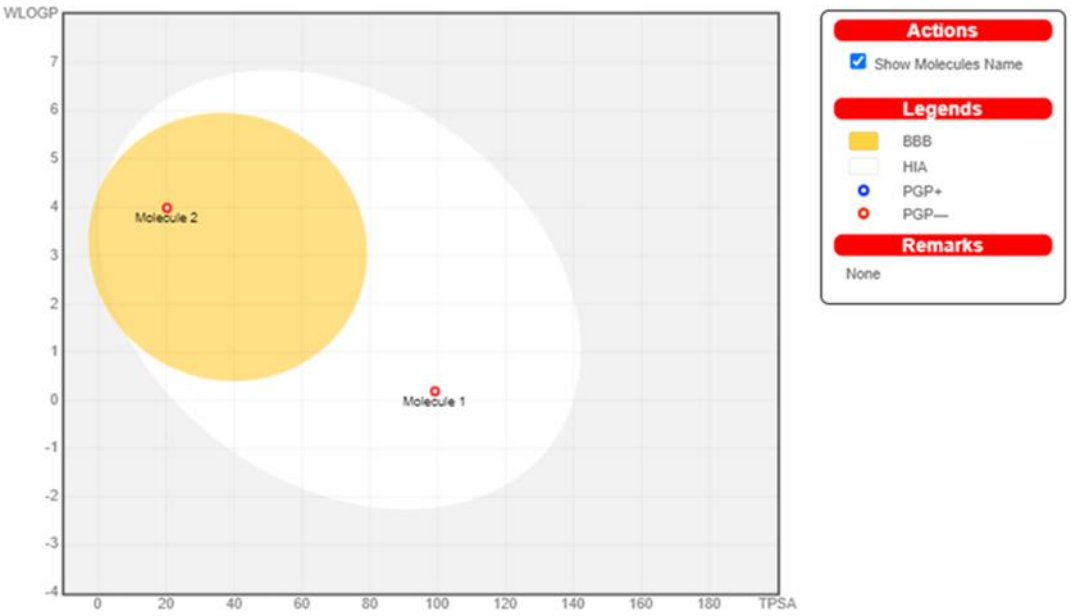
From the above observations it can be concluded that docking score of ligands Phenol, 3,5-bis(1,1-dimethylethyl) and triethyl citrate with proteins Microlysin, Glucansucrase, dipeptidyl peptidase and Cystalysin exhibited docking scores comparable to that of standard chlorhexidine.

Absorption Distribution Metabolism and Excretion

Brain Or IntestinaL EstimateD Egg (BOILED Egg) Pharmacological Absorption Property for prediction of GI absorption and prediction of blood brain barrier penetration plays an important role.

Brain Or IntestinaL EstimateD permeation method (BOILED-Egg) is proposed for two compounds to predict the passive gastrointestinal absorption and brain access. Triethyl citrate Yellow region (yolk) is the molecule predicted to be passively permeated through the blood-brain barrier (BBB), while Phenol, 3,5-bis(1,1-dimethylethyl)- in the white region is the molecule predicted to be passively absorbed by the gastrointestinal tract. (Figure 12)

Figure 12: BOILED Egg Model depicting gastrointestinal absorption of molecule 1(Phenol, 3,5-bis(1,1-dimethylethyl) and molecule 2 (triethyl citrate)



Formulation of Herbal mouth wash

The formulation of mouth wash was carried out using different concentrations of the active ingredient (ethanolic extract of *I marginata*) keeping other variables constant. The concentration of the extract was initially 10µg and later increased to 25µg,50µg and 100µg. (Table 14).

At 100µg concentration of ethanolic extract of *I marginata* statistically significant zone of inhibition was observed for *S.mutans, P.gingivalis, T.forsythia, T.denticola.* Hence a concentration of 100µg/mL of ethanolic extract of *I.marginata* was considered for formulation of mouth wash.

Table 14: Formulations of mouth wash with different concentrations of ethanolic extract of *I.marginata*

S.No	Ingredient	F1	F2	F3	F4
1	Ethanolic extract of *I.marginata*	10µg	25µg	50µg	100µg
2	Glycerol	50	50	50	50
3	Saccharin	20	20	20	20
4	Sorbitol	50	50	50	50
5	Distilled Water	1000 ml	1000 ml	1000 ml	1000 ml

Table 15: Final composition of mouth wash

Composition		Water	Final Concentration
Herbal Extract Lyophilised	0.1 g	100	100µg/mL
Glycerol	50g	100	0.005 g/mL
Saccharin	20	100	0.02 g/mL
Sorbitol	50 g	100	0.005 g/mL
Water		600	
Total volume	1000 mL		

0.1g of lyophilised herbal extract was used for the formulation of the mouth wash .50g of glycerol was added as a humectant,20g saccharin was used as sweetener. To preserve the moisture and sweetness sorbitol was added. 600ml of water was added to make up the volume to 1000ml.

Accelerated Stability Studies

The formulation was subjected to accelerated stability studies to determine the shelf life according to the ICH (International Council for Harmonisation of Technical Requirements of Pharmaceuticals for Human Use) guidelines for the following parameters.[20]

Properties	0 Month	1 St month	2nd month	3rd month
Colour	Pale Brown	Pale brown	Pale brown	Mild brown
Odour	Pleasant	Pleasant	Pleasant	Pleasant
Foam	++	++	+	+
Taste	Mild sweet	Mild sweet	Mild sweet	Mild sweet
Phase separation	Nil	Nil	Nil	Nil
PH	6.8	6.8	6.58	6.56
Turbidity NTU	0.25	0.24	0.24	0.36
Viscosity mpa	1.09	1.08	1.08	1.06
Sedimentation at 10,000 rpm	nil	Nil	Nil	trace
precipitation	Nil	Nil	Trace	Trace(+)
Homogeneity	good	Good	Good	Significant
specific gravity g/cm^3	1.08	1.06	1.06	1.02
Density g/mL	0.996	0.996	0.998	0.998
Phenol content mg/L	5.6	5.4	5.2	5.14
Surface tension	0.33	0.38	0.41	0.45

Properties	4TH Month	5th month	6TH MONTH
Colour	Mild brown	Mild brown	Mild brown
Odour	Pleasant	Pleasant	Moderately
Foam	+	+	-
Taste	Mild sweet	Mild sweet	Trace sour
Phase separation	Nil	Nil	+
PH	6.54	6.52	6.0
Turbidity NTU	0.36	0.37	0.38
Viscosity mpa	1.06	1.06	1.06
Sedimentation at 10,000 rpm	trace	trace	trace
precipitation	Trace(+)	Trace(+)	++
Homogeneity	Significant	Significant	moderate
specific gravity g/cm^3	1.028	1.026	1.02
Density g/mL	0.998	0.998	0.92
Phenol content mg/L	5.14	5.10	5.0
Surface tension	0.45	0.45	0.46

There were no observable physical or chemical changes seen on observing the sample for period of 6 months

Cytotoxicity of cells

In vitro determination of hemolysis is important to determine the cytotoxicity of newer drugs. RBC haemolysis assay was done to assess the cytotoxicity of the formulation.[21]

The Scientific Committee for Consumer Safety (SCCS) established the hemolysis test as a necessary test for human consumption product approval.[22] According to the American Society for Testing and Materials (ASTM), less than 5% hemolysis is considered null.[23] Above this limit and up to 10% is assumed as low, and beyond 10% is perceived as marked hemolysis. Hemolysis represents the rupture or alteration of the integrity of the red blood cell membrane, causing the release of hemoglobin.[24]

Table18: Hemolysis of different concentrations of mouthwash and control

Sample	OD	% of Hemolysis
Control	0.008	0
25	0.008	0
50	0.008	0
75	0.0081	1.25
100	0.0081	1.25
1% SDS	0.65	80.25

Heavy metal detection

Plant based formulations are becoming increasingly common for the treatment of several conditions. The World Health Organization recommends that the finished products be checked for their heavy metal concentrations.

Table 19: Maximum permissible levels and test result of heavy metals

S.No	Test Parameter	Maximum permissible levels mg/L	Test result mg/L
1	Cadmium	0.3	<0.1
2	Lead	10	<0.1
3	Arsenic	3	<0.1
4	Mercury	1	<0.1

The presence of heavy metals namely cadmium, lead, arsenic and mercury in the formulation was less than 0.1mg/L. The observed values were well within the prescribed values mentioned in the Ayurvedic Pharmacopoeia.[25]

Antibacterial Activity of freshly prepared mouth wash

Initial antibacterial activity tests performed with ethanolic extracts of *I.marginata* demonstrated significant antibacterial activity against *S.mutans, P.gingivalis, T.denticola and T.forsythia,* thereby indicating that an effective antibacterial mouth wash can be formulated. Also, the presence of phenolic compound phenol 3,5 -bis (1,1-dimethyl ethyl) may have contributed significantly to the antibacterial property in accordance to previous studies that have shown phenolic compounds are important secondary metabolites that contribute to its antimicrobial, and antioxidant property.[26]

Table 20: MIC of mouth wash formulation

Test Pathogen	MIC of freshly prepared mouth wash	MIC of mouth wash after 6 months	pvalue
S.mutans	7.25	15	≤0.001
P.gingivalis	100	100	1.000
T.denticola	100	100	1.000
T.forsythia	75	75	1.000

There was no statistically significant difference in MIC value of freshly prepared mouth wash and MIC of mouth wash after a period of 6 months. This observation suggests that there is no significant deterioration in the antibacterial property of the mouth wash against *S.mutans, T.denticola, T.forsythia* and *P.gingivalis.*

CONCLUSION

Based on the findings of this investigation, it can be deduced that the ethanolic extract of *I.marginata* exhibited demonstrable antibacterial activity against specific oral microorganisms that are known to be critical in the development of plaque, the primary etiologic factor for periodontitis and gingivitis. Mouthwashes made of natural ingredients, such as herbs and organic oils, are free of alcohol and chemical preservatives and offer special medicinal benefits.

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